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Accelerated Amyloid Deposition in the Brains of Transgenic Mice Coexpressing Mutant Presenilin 1 and Amyloid Precursor Proteins

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Summary

Missense mutations in two related genes, termed presenilin 1 (*PS1*) and presenilin 2 (*PS2*), cause dementia in a subset of early-onset familial Alzheimer's disease (FAD) pedigrees. In a variety of experimental in vitro and in vivo settings, FAD-linked presenilin variants influence the processing of the amyloid precursor protein (APP), leading to elevated levels of the highly fibrillogenic Aβ1–42 peptides that are preferentially deposited in the brains of Alzheimer Disease (AD) patients. In this report, we demonstrate that transgenic animals that coexpress an FAD-linked human PS1 variant (A246E) and a chimeric mouse/human APP harboring mutations linked to Swedish FAD kindreds (APP swe) develop numerous amyloid deposits much earlier than age-matched mice expressing APP swe and wild-type Hu PS1 or APP swe alone. These results provide evidence for the view that one pathogenic mechanism by which FAD-linked mutant PS1 causes AD is to accelerate the rate of β-amyloid deposition in brain.

Introduction

Alzheimer's disease (AD), the most common type of progressive dementia in the elderly, is characterized by neurofibrillary tangles and parenchymal deposits of β-amyloid (Aβ; Terry and Katzman, 1983). A subset of AD cases occur relatively early (onset in the fourth to sixth decade) and are familial (FAD) autosomal dominant disorders caused by mutations in the *presenilin 1* (*PS1*) gene on chromosome 14 (St. George-Hyslop et al., 1992; Alzheimer's Disease Collaborative Group, 1995; Chapman et al., 1995; Cruts et al., 1995; Perez-Tur et al., 1995; Sherrington et al., 1995; Wasco et al., 1995; Boteva et al., 1996; Campion et al., 1996; Sherrington et al., 1996), the *presenilin 2* (*PS2*) gene on chromosome 1 (Levy-Lahad et al., 1995; Rogaev et al., 1995), or the *amyloid precursor protein* (*APP*) gene on chromosome 21 (McKhann et al., 1984; Chartier-Harlin et al., 1991; Goate et al., 1991; Naruse et al., 1991). Approximately 30% of early-onset FAD pedigrees cosegregat with mutations in *PS1* (Sch ll nb rg, 1995).

β-amyloid (Aβ) p ptides are ndoproteolytically generated from the amyloid precursor protein (APP; Haass et al., 1992; Shoji et al., 1992), an integral membrane protein (Weidemann et al., 1989), and FAD-linked mutations in APP increase the amount, length, or fibrillogenic properties of Aβ species (Wisniewski et al., 1991; Citron et al., 1992; Cai et al., 1993; Haass et al., 1994; Suzuki et al., 1994). Recent studies indicate that FAD mutant PS1 alters APP processing to enhance the generati n of Aβ1–42 peptides, which appear to be more fibrillogenic than Aβ1–40 (Jarrett et al., 1993). Patient plasma, as well as conditioned medium of fibroblast cultures fr m patients with FAD-linked PS1 and PS2 variants (Scheuner et al., 1996) and conditioned medium from transfect d mammalian cells expressing mutant PS1 and PS2 variants (Borchelt et al., 1996b; Duff et al., 1996; Citron et al., 1997; Tomita et al., 1997), have been shown to contain higher levels of Aβ1–42 than relevant controls. To examine the impact of mutant PS1 on Aβ1–42 levels in an in vivo setting, we produced transgenic mice that coexpress FAD mutant human (Hu) PS1-A246E and a chim - ric mouse/human (Mo/Hu) APP695 harboring a Hu Aβ domain and mutations (K595N, M596L; Mullan et al., 1992) linked to Swedish FAD pedigrees (APP swe; Borchelt et al., 1996b). In comparison to transgenic mic expressing APP swe alone or doubly transgenic mic coexpressing wild-type Hu PS1 and APP swe, mice coexpressing Hu PS1-A246E and APP swe contained higher concentrations of Aβ1–42 in brain tissue (Borch lt et al., 1996b). Similarly, mutant PS1 selectively influences the processing of endogenous mouse APP and wild-type human APP to increase production of Aβ1–42 in transgenic mouse brains (Duff et al., 1996; Citron et al., 1997).

In the present report, we examine the extent and frequency of Aβ deposits in 12-month-old transgenic mice that coexpress Hu PS1-A246E and APP swe, mice that coexpress wild-type Hu PS1 and APP swe, mice that express APP swe alone, and mice that express mutant PS1 alone. We demonstrate that only mice coexpressing Hu PS1-A246E and APP swe develop Aβ deposits by 12 months of age. Moreover, we demonstrate that co x- pression of Hu PS1-A246E with APP swe reduces th interval of the formation of initial Aβ deposits from 18 months for APP swe alone to 9 months for APP sw with Hu PS1-A246E. These data provide strong evidence in support of the hypothesis that a principal pathway by which mutations in PS1 predispose individuals to FAD is to accelerate Aβ deposition.

Results

To test directly whether the expression of FAD mutant PS1 promotes amyloid deposition, we examined th brains of aged (12-month-old) transgenic mice that co xpress Hu PS1-A246E and APP sw . In previ us xaminations of young r (2- to 3-m nth-old) mic x- pressing th se transgen s, w dem nstrated that th

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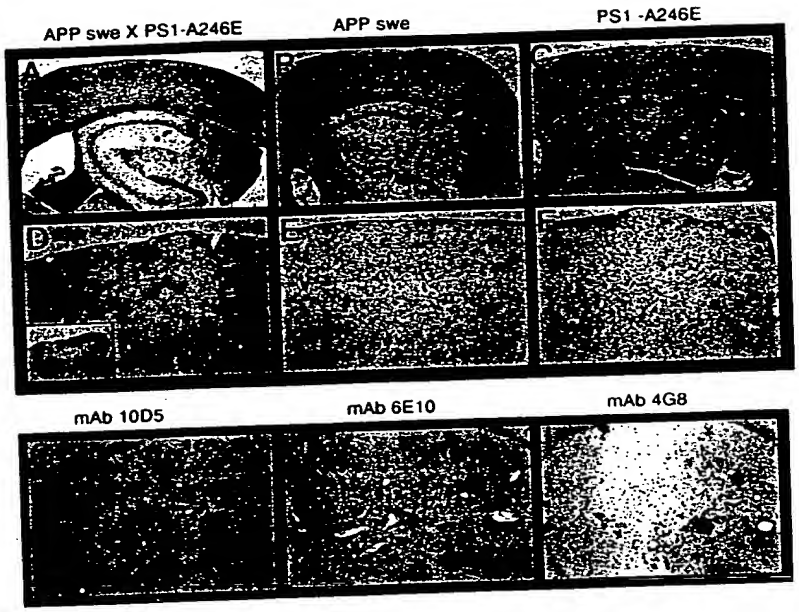

Figure 1. Mice Coexpressing Hu PS1-A246E and APP swe Develop Aβ Deposits

(A–F) Twelve-month-old transgenic mice that coexpress Hu PS1-A246E and APP swe ([A] and [D]), APP swe alone ([B] and [E]), and Hu PS1-A246E alone ([C] and [F]) were examined by immunocytochemical staining for Aβ peptides with the monoclonal antibody 10D5.

(A–C) Hippocampus and occipital cortex (magnification, 8×).

(D–F) Frontal cortex (magnification, 20×; inset in [D], 8×).

(G–I) Sections (magnification, 20×) of hippocampus from 12-month-old mice coexpressing Hu PS1-A246E and APP swe stained with 10D5 (G), 6E10 (H), and 4G8 (I). The 4G8 antibody appears to preferentially recognize the more compact deposits.

rati of Aβ1–42/43 to Aβ1–40 is elevated by ~50% in doubly transgenic mice expressing APP swe and Hu PS1-A246E as compared with transgenic mice expressing APP swe alone or doubly transgenic mice coexpr ssing wild-type Hu PS1 and APP swe (Borchelt et al., 1996b).

Brain tissues from animals that coexpress Hu PS1-A246E (line N-5) and APP swe (line C3–3) were stained with Aβ monoclonal antibodies (mAb) 10D5 (Games et al., 1995), 4G8, and 6E10 (Kim et al., 1990); the latter antibody is specific for Hu Aβ (Kim et al., 1990; Borchelt t al., 1996a). The hippocampus (Figures 1A and 1G–1I) and frontal cortex (Figure 1D) of these animals invariably c ntained numerous amyloid deposits that were immunoreactive with all three antibodies (Figure 1G, 10D5; Figure 1H, 6E10; Figure 1I, 4G8). The brains from age-matched littermates expressing APP695 swe alone (Figures 1B and 1E) or from littermates expressing Hu PS1-A246E alone (Figures 1C and 1F) were free of Aβ deposits. Aβ deposits (Figure 2A) were also detected by conventional silver impregnation methods (Nakano and Hirano, 1987; Figure 2B). Many of the large argentophilic amyloid deposits contained dystrophic neurite profiles (Figure 2B, arrowheads) that also showed APP immunoreactivity with the APP-specific antibody CT15 (Sisodia et al., 1993; Borchelt et al., 1994), a reagent that has previously been used to visualize dystrophic n urites in aged nonhuman primates (Martin et al., 1994). Many deposits (Figure 2D) were also associated with reactive astrocytes as revealed by immunostaining with antibodies to glial fibrillary acidic protein (GFAP; Figure 2E). Notably, nuclei with the features normally ass ciated with microglia were present in some of the larger deposits (see Figure 2C). Thus, these data demonstrate, by multiple criteria (presence of Aβ immunoreactivity, argentophilic deposits, neuritic profiles, reactive astrocyt sis), that animals c expressing APP sw and Hu PS1-A246E d velop Aβ deposits that r sembl those found in AD brain and in aged nonhuman primates (Martin et al., 1994).

Wild-Type Hu PS1 Does Not Accelerate Aβ Deposition

To determine whether the acceleration in Aβ deposition was due solely to the coexpression of Hu PS1 with APP swe, we examined the brains of age-matched 12-month-old animals coexpressing APP swe (line C3–3) and wild-type Hu PS1 (line S8–4; Figure 3). We document that, in contrast to mice coexpressing APP swe and Hu PS1-A246E, 12-month-old mice coexpressing APP swe and wild-type Hu PS1 were free of Aβ deposits (Figures 3B and 3D). Notably, previous characterizations of wild-type Hu PS1 mRNA expression in line S8–4 demonstrated that the levels of transgene-derived mRNA are approximately fivefold higher in the S8–4 line than the N-5 line, which expresses Hu PS1-A246E (Thinakaran et al., 1996; Lee et al., 1997). Thus, even when expressed at considerably higher levels than FAD-linked mutant PS1, wild-type Hu PS1 neither elevates Aβ1–42 production (Borchelt et al., 1996b) nor promotes Aβ deposition.

FAD-Linked Hu PS1-A246E Dramatically Accelerates the Rate of Aβ Deposition

To determine the degree to which coexpression of Hu PS1-A246E with APP swe accelerated the rate of amyloid deposition, we compared the extent and frequency of Aβ deposits in 9-, 10-, and 11-month-old animals coexpressing APP swe and Hu PS1-A246E to that of animals (of varied ages) expressing APP swe alone (Figure 4; Table 1). The number of Aβ deposits in 9-month-old animals coexpressing APP swe and Hu PS1-A246E were comparable to 18-month-old animals expressing APP swe alone (compare Figures 4A and 4D; Table 1). The frequency of Aβ deposits in mice coexpressing APP sw and Hu PS1-A246E incr ased dramatically between 10 and 12 months of ag (Figures 4B and 4C; Tabl 1),

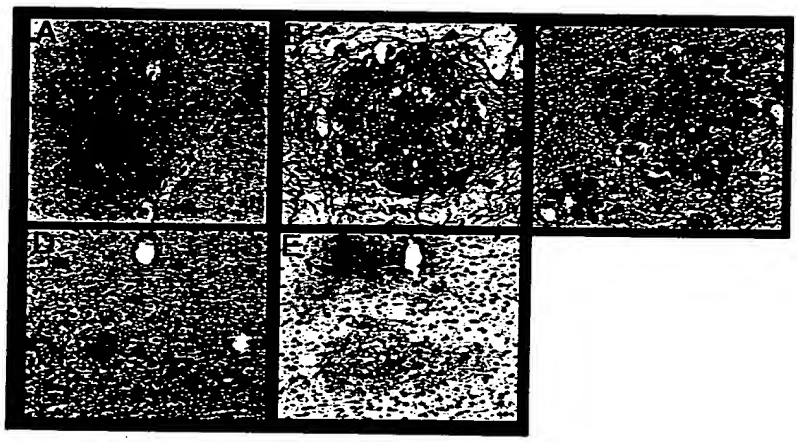

Figure 2. Mice Coexpressing Hu PS1-A246E and APP swe Develop Amyloid Deposits and Dystrophic Neurites Associated with Reactive Gliosis

Twelve-month-old mice doubly transgenic for Hu PS1-A246E and APP swe were examined by silver impregnation methods (Nakano and Hirano, 1987) and immunostaining for Aβ (10D5), APP C-terminal epitopes (CT15), and GFAP.

(A) A compact Aβ deposit in the hippocampus immunostained with the 10D5 antibody.

(B) A large Aβ deposit in the hippocampus with neurites stained by silver impregnation methods (Nakano and Hirano, 1987); the dystrophic neurites are marked by arrowheads.

(C) A large plaque in the hippocampus stained with APP-specific CT-15 antisera (Sisodia et al., 1993; Borchelt et al., 1994).

(D) Aβ deposits in the occipital cortex stained by silver impregnation methods.

(E) The section adjacent to that shown in (D), stained with anti-GFAP antibodies.

far exceeding the number of deposits in much older mice expressing APP swe alone. Collectively, these data convincingly demonstrate that the Hu PS1-A246E acts synergistically with APP swe to accelerate amyloid deposition.

In our analyses of younger 9- and 10-month-old animals coexpressing APP swe and Hu PS1-A246E, it appeared that the initial deposits were most frequently observed in the hippocampus near the ventricles, in the subiculum, and in the occipital and frontal cortices. Even up to the age of 12 months, Aβ deposits have not been detected in any region of the brain other than the cortex and hippocampus (data not shown), despite relatively high levels of expression of the transgene throughout the brain (Borchelt et al., 1996a). Moreover, examinations of brain tissues from 9- and 12-month-old mic expressing Hu PS1-A246E alone (n = 4) showed normal

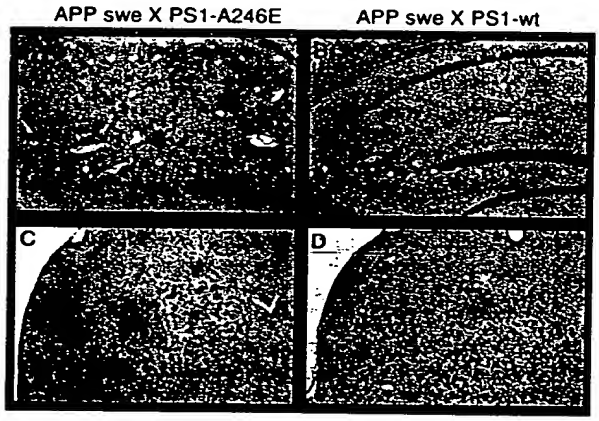

Figure 3. Aβ Deposits Do Not Occur in 12-Month-Old Mice Coexpressing APP swe and Wild-Type Hu PS1

The brains of three 12-month-old and two 11-month-old mice coexpressing APP swe and wild-type Hu PS1 were immunostained with mAb 6E10 for Aβ deposits.

(A and C) Hippocampus and frontal cortex, respectively, of a 12-month-old animal coexpressing APP swe and Hu PS1-A246E.

(B and D) Hippocampus and frontal cortex of a 12-month-old animal coexpressing APP swe and wild-type Hu PS1.

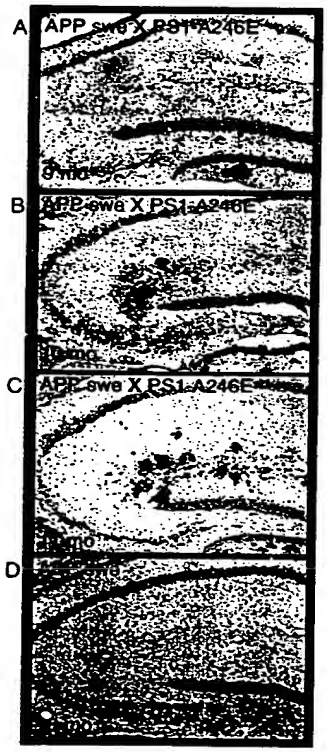

Figure 4. The Coexpression of Hu PS1-A246E with APP swe Greatly Accelerates the Rate of Aβ Deposition

The brains of 9-, 10-, and 11-month-old animals coexpressing APP swe and Hu PS1-A246E were compared to that of 18-month-old animals expressing APP swe alone (these 18-month-old APP swe mice were the dams of the double APP swe × PS1 transgenic animals). All sections were immunostained with mAb 6E10.

Table 1. The Expression of Hu PS1-A246E Greatly Accelerates the Deposition of Aβ in Mice Harboring APP swe Transgenes

Genotype	n	Age	AB Deposits
APP swe × Hu PS1-A246E	2	9 months	+
	2	10 months	++
	2	11 months	+++
	4	12 months	+++
APP swe × wild-type Hu PS1	2	11 months	−
	3	12 months	−
Hu PS1-A246E	2*	12 months	−
	2*	9 months	−
APP swe	1*	10 months	−
	3*	12 months	−
	2†	12 months	−
	1†	14 months	−
	4‡	18 months	+
	1†	20 months	+

Because assigning numerical values to Aβ-deposit loads is somewhat subjective, we chose to use an arbitrary unit system to classify the relative Aβ deposit loads of various animals. Animals with low numbers of Aβ deposits per sagittal section through the cortex and hippocampus, as exemplified by the 9-month-old APP swe × Hu PS1-A246E (Figure 4A) were assigned a single (+). Animals with 5–20 Aβ deposits per section (not counting small satellite deposits that surround the large neuritic deposits) were assigned (++), and animals with >20 Aβ deposits per section (not counting satellites) were assigned (+++). APP swe and Hu PS1-A246E singly transgenic animals labeled with * are littermates from the breedings of APP swe mice with the PS1 mice. APP swe animals labeled with † are from a separate breeding colony of C3-3 APP swe mice in hybrid backgrounds of C3H/HeJ and C57BL/6J mice. APP swe animals labeled with ‡ are the APP swe transgenic dams ([C3H/HeJ × C57BL/6J F3] × C57BL/6J n1) of all double transgenic litters.

patterns of cytology. The numbers of cortical and hippocampal neurons were not obviously diminished, and neuritic abnormalities and reactive astrogliosis were not observed (data not shown). Thus, the presence of the FAD-linked mutant Hu PS1-A246E appears to have no obvious impact on the mouse nervous system, apart from its influence on amyloid deposition in cortical and hippocampal regions.

Aβ Deposits in Mice Coexpressing Hu PS1-A246E and APP swe Contain Both Aβ1–40 and 1–42 Epitopes

To characterize the Aβ deposits in mice coexpressing Hu PS1-A246E and APP swe, tissue sections were stained with antisera Ab40 and Ab42, which are specific for Aβ peptides terminating at amino acids 40 or 42, respectively (Quality Control Biochemicals, Hopkinton, MA). As previously reported, Aβ deposits in human brain were preferentially immunoreactive with the Ab42 antiserum (Figure 5A; Iwatsubo et al., 1994, 1995, 1996; Gravina et al., 1995; Lemere et al., 1996), and this immunoreactivity was blocked by synthetic Aβ1–42 peptides (Figure 5B). In AD brain, Ab40 appeared to recognize only a subset of the very densely packed Aβ deposits (Figures 5C and 5D). In contrast, the majority of amyloid deposits in both the cortex and hippocampus of the Hu PS1-A246E × APP swe transgenic mice showed Ab40 immunoreactivity (compare Figures 5E and 5G). The immunoreactivity of both antibodies in the brains of these transgenic mice was completely blocked by the relevant peptide antigen (Figures 5F and 5H). Interestingly, in the sections from mouse brain, the numbers of Ab40 and Ab42 immunoreactive deposits were lower than predicted from 6E10 immunostaining or silver staining. Moreover, both the Ab42 and Ab40 antibodies seemed to be most reactive with the dense core structures in the center of the deposit. To confirm the Ab42 and Ab40 immunocytochemical studies, we obtained another set of rabbit polyclonal antibodies that are specific for Aβ1–40 and Aβ1–42 (3540 and 3542, respectively) from Drs. Heline Barelli and Frederic Checler (Institute of Molecular and Cellular Pharmacology, Valbonne, France; Barelli et al., 1997). Using these latter reagents, the patterns of immunoreactivity in human AD brain and the double transgenic mice were indistinguishable from the patterns observed with Ab40 and Ab42.

Discussion

This report demonstrates that the coexpression of APP swe with Hu PS1-A246E, but not wild-type Hu PS1, greatly accelerates the rate of Aβ deposition in the brains of transgenic mice. These new data extend our earlier studies of younger littermates that coexpress APP swe and Hu PS1-A246E, in which we demonstrated that only mutant PS1 influences APP processing in a manner that leads to increased concentrations of Aβ1–42 in brain (Borchelt et al., 1996b). However, it is not entirely clear whether the accelerated rate of Aβ deposition that occurs in the APP swe × Hu PS1-A246E mice is due solely to increases in the levels of Aβ1–42. Based on in vitro aggregation studies (Jarrett et al., 1993), which show Aβ1–42 to be more fibrillogenic, and immunocytochemical studies of brains from sporadic AD (Iwatsubo et al., 1994; Gravina et al., 1995), Swedish kindreds of familial AD (Mann et al., 1996), and Down's Syndrome (Iwatsubo et al., 1995; Lemere et al., 1996), which show that Aβ1–42 is deposited early and selectively, we predicted that the Aβ deposits occurring in our double transgenic mice would be immunoreactive with antibodies to Aβ1–42 (Borchelt et al., 1996b). However, we discovered that the majority of Aβ deposits in our double transgenic mice were not immunoreactive with either of two polyclonal antisera to the C-terminus

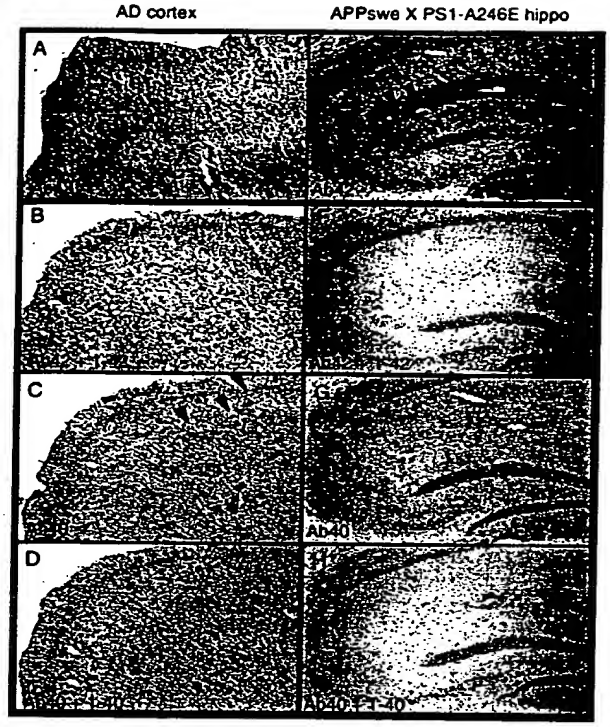

Figure 5. Aβ1–40 and 1–42 Are Codeposited in the Brains of APP swe × Hu PS1-A246E Mice

Antibodies specific to the C-terminus of Aβ1–40 (Ab40) and 1–42 (Ab42) were used to examine the relative deposition of Aβ1–40 and 1–42 in the brains of 12-month-old mice coexpressing APP swe and Hu PS1-A246E. As a control, sections from the cortex of an end-stage AD case were immunostained in parallel. Antibody specificity was demonstrated by preincubating the antibody overnight in Tris-buffered saline with 10 μg/ml of peptide antigen. Notably, in addition to the plaque immunostaining detected by Ab42, many neuronal profiles appeared to be specifically stained (immunoreactivity was completely competed with relevant peptide antigen). The significance of this immunostaining is not clear at present, as no other Aβ antibody produced a similar pattern of immunostaining.

of Aβ1–42 (Ab42 and 3542) but instead were stained with antisera to Aβ1–40 (Ab40 and 3540). All immunoreactivity with both sets of antibodies could be completely competed with the relevant peptide antigen. Importantly, our analyses of human AD brain, using the polyclonal antibodies to Aβ1–42 peptides, revealed large numbers of Aβ deposits, whereas the Aβ1–40 antibodies recognized relatively few deposits; these results are fully consistent with earlier immunocytochemical studies with a variety of monoclonal antibodies (Iwatsubo et al., 1994, 1995; Gravina et al., 1995; Lemere et al., 1996). Thus, we believe that the antibodies used in our study are selectively reactive with the C-termini of Aβ1–40 and Aβ1–42.

We envision several scenarios to explain our Aβ1–40 and 1–42 immunoreactivity data in the mice. First, Aβ1–40 may be deposited more readily in mice; deposition of Aβ1–40 was observed in mice expressing high levels of Hu APP695 with the Swedish mutations (Hsiao et al., 1996). However, this scenario does not explain why the majority of deposits in our double transgenic mice were not recognized by antibodies to Aβ1–42. Second, it is possible that the Ab42 antibody discriminately recognizes a specific conformation of Aβ*–42 fibrils. However, all tissues are denatured in 70% formic acid prior to immunostaining. Third, Aβ1–42 fibrils may, in mice, form structures that are highly resistant to denaturants, thus precluding detection. Finally, exoproteolytic activities in mouse brain may degrade the deposited Aβ1–42 peptides to reveal the Ab40 epitope (which is removed more slowly). Consistent with the latter two scenarios, Ab40 and Ab42 antisera recognized only ~70% and 20%, respectively, of the available Aβ deposits. Moreover, both antibodies appeared to preferentially reveal only the most densely packed core of the deposit. Thus, it is not presently clear whether the relative frequency and intensity of immunostaining with the Ab40 and Ab42 antibodies accurately reflects antigen abundance or antigen availability.

Although it is not clear whether increased Aβ1–42 is the only driving force behind the accelerated Aβ deposition that occurred in our double transgenic mice, our new findings add to the growing body of evidence supporting the view (Selkoe, 1997) that amyloid deposition is an early and critical event in the pathogenesis of AD. Previous studies have established that mutations in APP occur in kindreds of familial AD (McKhann et al., 1984; Chartier-Harlin et al., 1991; Goate et al., 1991; Narus et al., 1991) and that these mutations all alter proteolytic processing to favor the formation of Aβ1–42 peptides (Citron et al., 1992; Cai et al., 1993; Haass et al., 1994; Suzuki et al., 1994). More recently, it has become clear that FAD-linked PS1 variants influence APP processing to promote the production of Aβ1–42/43 peptides (Borchelt et al., 1996b; Duff et al., 1996; Citron et al., 1997). Finally, immunocytochemical studies of brains from patients with AD (Iwatsubo et al., 1994; Gravina et al., 1995) and Down's Syndrome (Iwatsubo et al., 1995; Lemere et al., 1996) indicate that Aβ1–42 deposition is an early event in disease pathogenesis. Adding to this large body of evidence, we now demonstrate that mutant PS1 acts synergistically with mutant APP to markedly accelerate Aβ deposition in the CNS of transgenic mice. Collectively, these findings support the view that Aβ deposition is an early and significant event in the pathogenesis of AD.

Experimental Procedures

Transgenic Mice

Most of the transgenic animals used in the present study are older littermates of animals used in previous examinations of Aβ peptide levels (Borchelt et al., 1996b). All four genotypes examined (APP swe × Hu PS1-A246E; APP swe × wild-type Hu PS1, APP swe only, and Hu PS1-A246E only) were represented in offspring that were generated from matings between APP swe transgenic mice (strain background = [C3H/HeJ × C57BL/6J F3] × C57BL/6J n1) and Hu PS1-A246E transgenic mice (strain background = C3H/HeJ × C57BL/6J F3). A subset of the older APP swe transgenic mice were from separate matings maintained in C3H/HeJ × C57BL/6J hybrid backgrounds. All animals were maintained in pathogen-free microisolator cages.

Immunoctyochemistry and Histology

Twelve-month-old transgenic mice that coexpress wild-type or Hu PS1-A246E and APP swe, express APP swe alone, and express Hu

PS1-A246E alone were perfused with 1× Dulbecco's phosphate-buffered saline (pH 7.1; Gibco BRL, Life Technologies, Grand Island, NY) followed by 4% paraformaldehyde, buffered with 1× Dulbecco's phosphate-buffered saline (pH 7.1). Immunostaining with mAb 10D5, 6E10, and 4G8 was performed on paraffin-embedded, 10 μm sections (supported on Vectabond-coated slides). Immunostaining with the rabbit polyclonal antibodies Ab40 and Ab42 was performed on free-floating frozen sections. Prior to immunostaining, paraffin-embedded sections were deparaffinized by oven heating, followed by a 3 min incubation in 70% formic acid (longer incubations diminish tissue integrity) and further deparaffinization in xylene followed by washes in 100% ethanol, 95% ethanol, 70% ethanol, and water. Endogenous peroxidase activities were quenched by 30 min incubations in 3% H_2O_2 (in methanol) before sections were microwaved for 5–7 min in water, cooled for 5 min, rinsed with water, and then washed in Tris-buffered saline (TBS; 0.05 M Tris-Cl [pH 7.6] and 0.25 M NaCl). All free-floating sections stained for Aβ were treated with 70% formic acid for 3 min prior to immunostaining. Nonspecific epitopes were then blocked with 3% normal goat serum and 0.1% Triton X-100 in TBS. After 1 hr of blocking, primary antibodies Ab40 (Quality Control Biochemicals, Hopkinton, MA), Ab42 (Quality Control Biochemicals, Hopkinton, MA), 10D5 (a gift from Dr. Dale Shenk, Athena Neurosciences, South San Francisco, CA), 6E10 (Kim et al., 1990), or 4G8 (Kim et al., 1990) were diluted to a final concentration of 5 μg/ml in TBS with 2% normal goat serum and incubated overnight at 4°C in a humid chamber. Sections were then washed in TBS 3 times for 5 min each and incubated with secondary antibodies and peroxidase-coupled streptavidin as described by the manufacturer (Vectastain ABC Kit; Vector Laboratories, Incorporated, Burlingame, CA). All sections were lightly counter-stained with hematoxylin and eosin, following standard histological procedures.

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